A mecC allotype, mecC3, in the CoNS Staphylococcus caeli, encoded within a variant SCCmecC

A *mecC* allotype, *mecC*3, in the coagulase-negative staphylococcus *Staphylococcus caeli*, encoded within a variant SCC*mecC*

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Running title

A novel *mecC* allotype in a new species of staphylococci.

Synopsis

Objectives

Methicillin-resistance in staphylococci is conferred by an alternative penicillin-binding protein (PBP2a/2') with low affinity for most β-lactam antibiotics. PBP2a is encoded by *mecA* which is carried on a mobile genetic element known as SCC*mec*. A variant of *mecA*, *mecC*, was described in 2011 and has been found in *Staphylococcus aureus* from humans and a wide range of animal species as well as a small number of other staphylococcal species from animals. In this study we characterise a novel *mecC* allotype, *mecC*3, encoded by an environmental isolate of *Staphylococcus caeli* cultured from air sampling of a commercial rabbit holding.

Methods

The *S*. *caeli* isolate, 82B^{T} was collected in Italy in 2013 and genome sequenced using MiSeq technology. This allowed the assembly and comparative genomic study of the novel SCC*mec* region encoding *mecC*3.

Results

The study isolate encodes a novel *mecA* allotype, *mecC*3 with 92% nucleotide identity to *mecC*. *mecC*3 is encoded within a novel SCC*mec* element distinct from those previously associated with *mecC* including a *ccrAB* pairing (*ccrA5B3*) not previously linked to *mecC*.

Conclusions

Our study is the first description of the novel *mecC* allotype *mecC*3, the first isolation of a *mecC*-positive staphylococcus in Italy and the first report of *mecC* in *S*. *caeli*. Furthermore, the SCC*mec* element described here is highly dissimilar to the archetypal SCC*mec* XI encoding *mecC* in *S*. *aureus* and to elements encoding *mecC* in other staphylococci. Together our report highlights the

diversity of *mecC* allotypes and the diverse staphylococcal species, ecological settings and genomic context in which *mecC* may be found.

Key words: staphylococci, methicillin-resistance, *mecC*, *Staphylococcus caeli,*

Introduction

Methicillin-resistance in *Staphylocccus aureus* is typically conferred by the gene *mecA* along with two variants, *mecB* and *mecC*. [1-4] *mec* gene resistance is mediated by an alternative penicillin-binding protein with reduced affinity for almost all β-lactam antimicrobials. [5, 6] Since its first discovery in bulk tank milk on an English dairy farm, [1] *mecC* has been found in *S*. *aureus* isolates from a wide range of host species, including human carriage and infection and various wildlife, companion and livestock species [7, 8] with genomic analysis indicating zoonotic transmission from livestock to humans. [9, 10] *mecC*-MRSA have been reported from a range of countries and while this geographical distribution has centred on Europe [8] it has also been reported in Australia. [11] In addition to *S*. *aureus*, *mecC* has been described in other species of staphylococci, albeit in only a limited number of species and a small number of isolates which have all come from animals: *Staphylococcus xylosus*, [12] *Staphylococcus sciuri*, [13] *Staphylococcus stepanovicci* [14] and *Staphylococcus saprophyticus* [15, 16] or in the case of *Staphylococcus edaphicus* from environmental sampling. [17] *mecC* in MRSA is found within a staphylococcal cassette chromosome *mec* (SCC*mec*) type XI while in other staphylococci it is found in a range of genomic contexts, although always within the *orfX* region and with features common to SCC*mec* elements. Divergent *mecC* allotypes *mecC1* and *mecC2* have been described in *S*. *xylosus* [12] and *S*. *saprophyticus*, [15] respectively but have not been reported in *S*. *aureus*, suggesting a greater diversity and ancestral association of *mecC* with non-*aureus* staphylococci.

Here we describe a novel *mecC* allotype, *mecC*3, encoded within a distinct and novel SCC*mec* element in a newly described species, *Staphlyococcus caeli* [18] that furthers our understanding of the diversity of *mecC* genes and the diverse species and genetic elements that carry it.

Materials and methods

Isolate collection and whole genome sequencing

The *S. caeli* isolate, 82B^T (=NCTC 14063^T =CCUG 71912^T), was collected from air sampling in a commercial rabbit holding in Italy in 2013 as part of a previous study [19] and has been described elsewhere, including its genome sequencing. [18] MRSA was also present on the sampled farm. [19] Six months prior to the isolation of 82B^T, ST398 MRSA belonging to t034 and t5210 were isolated from farm workers, their relatives and rabbits on the farms. [19] At the time of 82^T isolation MRSA belonging to t034, t5210, t1190 and t2970 were isolated from rabbits and humans with non-typed MRSA isolates found in air samples and surface swabs. [19]

Sequence assembly and identification of SCC*mec*

Sequencing reads were *de novo* assembled using Velvet [20] and annotated using the NCBI Prokaryotic Genome Annotation Pipeline. [21] Contiguous sequences (contigs) containing the *orfX* and *mecC* genes, were identified using BLAST, using the respective genes from *S. aureus* LGA251 (accession number, FR821779) as query sequences. Contigs NZ_FMPG01000005.1 and NZ_FMPG01000008.1 were identified as containing the *orfX* and *mecC* genes, respectively. Primers were designed for the end of contig NZ_FMPG01000005.1 and start of contig NZ_FMPG01000008.1, followed by PCR, with the resulting amplicon being ABI sequenced (Source Bioscience, Cambridge, UK), in order to close the gap between the contigs. Primers were designed using Primer3 (http://primer3.ut.ee/).

Phylogenetic analysis of *mecA* homologues

Phylogenetic analyses were carried out in MEGA7. [22] All nucleotide sequences were obtained from NCBI databases, using the following accession numbers; *S. aureus* LGA251, FR821779, *mecC*; *S. xylosus* S04009, HE993884, *mecC1*; *S. saprophyticus* 210, KF955540, *mecC2*, *S. caeli* 82B, *mecC*3, FMPG01000008; *S. sciuri* K11, Y13094, *mecA1; S. aureus* N315, D86923, *mecA*;

S. vitulinus CSBO8, AM048810, *mecA2*; *M. caseolyticus* IMD0819, KY013611, *mecD*; *M. caseolyticus* JCSC5402, NC_011996, *mecB*. The sequences were aligned using MUSCLE and a Maximum Likelihood tree was generated, using a General Time Reversible model. Site substitution rates were calculated using a discrete gamma distribution model.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by Vitek2 using AST P260 cards following the manufacturer's instructions. Disc diffusion was performed following EUCAST disk diffusion method Version 6.0 January 2017. Growth on MRSA Brilliance (Oxoid) was tested by spreading a 1 µl loop taken from a 0.5 McF suspension (~1.5 x 10^5 cfu) onto the Brilliance plate and incubated at 30°C, 35°C and 37°C for 24 hours. Interpretation was done based on the criteria for coagulase-negative staphylococci with *S. aureus* NCTC12497 and NCTC12493 used as control strains for susceptibility testing. PBP2a detection was performed using the PBP2' Latex Agglutination Test Kit (Oxoid, Baskingstoke, UK) following the manufacturer's instructions.

Results and Discussion

S. caeli 82B^T contains a novel *mecC* allotype, *mecC*3

Isolate 82B^T has been described as the type strain of a novel staphylococcal species, *S. caeli*, most closely related to *S. xylosus*, *S. saprophyticus* and *S. edaphicus*. [18] As part of the characterisation of 82B^T it was whole genome sequenced. Further investigation of the 82B^T genome by BLAST analysis using the *mecC* gene from *S. aureus* LGA251 as the query sequence revealed that 82B^T encodes a *mecC* homologue on contig NZ_FMPG01000008.1. Alignments of the 82B^T *mecC* gene with known *mecC* allotypes showed that it shares 92% nucleotide identity with *mecC* from LGA251, 93% with *mecC1* from *S. xylosus* S04009, and 94% with *mecC2* from *S. saprophyticus* 210. Based on these results and in line with the guidelines for the classification for *mecA* homologues [23], the *mecC* gene of *S. caeli* 82B^T is a new allotype of *mecC*, and therefore designated *mecC*3. The phylogenetic relationship of *mecC*3 to the other *mecA* homologues is illustrated in Figure 1, this suggests that the more common *mecC* has evolved from the more ancestral forms (*mecC1*, *mecC2*, *mecC*3) and that of the three, *mecC*3 is closest to the ancestral form. The *mecC* complex of *S. caeli* 82B^T shares 93% and 92% nucleotide identity with the complete *mecC* complex of LGA251 and S04009, respectively, with the partial *mecC* complex of *S. saprophyticus* 210 sharing 94%. 82B^T gave a negative reaction using the commercial PBP2a latex agglutination test kit from Oxoid. This reaction was not altered after cefoxitin induction. *mecC*3 in 82B^T was however detected by the published *mecC* primers pairs 1A and 1B [24] and mecC-Uni-F and mecC-Uni-R [13].

S. caeli 82B^T *mecC*3 is encoded within a distinct SCC*mec*-like element

mecC genes are typically found within SCC*mec* elements, [13, 14] which insert into the genome at the 3' end of a 23S rRNA methyltransferase gene, often referred to as *orfX*. [25, 26] BLASTn analysis using *orfX* from LGA251 as the query sequence identified the *orfX* region of 82B^T within contig

NZ_FMPG01000005.1. PCR and amplicon sequencing confirmed that the two contigs NZ_FMPG01000005.1 containing *orfX* and NZ_FMPG01000008.1 containing *mecC*3 are contiguous in the 82B^T genome with a gap of 140 bp between the ends of the two contigs. The assembled sequence generated in this study from these two contigs, encoding the *orfX* region of 82B^T and *mecC*3 including a SCC*mec*-like element has been deposited in NCBI with accession MH155596.

The integration site on the genome for SCC*mec* elements within *orfX*, known as the attachment (*attB*) site, is identified by the recombinase CcrAB/CcrC via a 14 bp sequence [27]. Insertion into the *attB* generally results in the SCC*mec* being flanked by direct repeats, *attR* and *attL* [13, 27, 28]. We therefore searched for these repeat sequences within the *orfX* region of 82B^T to define the limits of its putative SCC*mec*-like element. The search sequences; gc[ag]tatca[tc]aaatgatgcggttt and aacc[tg]catca[tc][tc][at][ac]c[tc]gataag[ct], produced from previously identified *attR* and *attL* sequences, respectively, identified an *attR* site 51 Kbp downstream of *orfX* and an *attL* site 3.8 Kbp downstream of *mecC*3 (Figure 2A). This indicates a SCC*mec* in 82B^T which is 127 Kbp in size, with the *orfX* and *mecC*3 regions at opposite ends of the element (Figure 2A). However, it is not clear if this represents a single entity or a composite element generated by consecutive insertions. Within this SCC*mec* region, 82B^T contains two *ccr* complexes and three joining (J) regions (Figure 2A). The *ccrA* and *ccrB* genes of 82B^T share 90% and 91% nucleotide identity with *ccrA5* and *ccrB3,* respectively, from *S. pseudintermedius* KM241 (accession number: AM904731). This represents a type 6 *ccr* allotype [25, 28], which is novel for a SCC*mec* encoding a *mecC*. Indeed this pairing is rare among SCC*mec* in staphylococci with a search of NCBI finding only two other staphylococcal isolates that contained this *ccrAB* pairing, *S. cohnii* WC28 (accession number: GU370073) [29] and *S. cohnii* SNUDS-2 (CP019597; locus tag BZ166_04625 and BZ166_04620). In addition to the *ccrAB* genes described above, the SCC*mec* of 82B^T also contains a *ccrC* gene, sharing 90% nucleotide identity with *S. aureus* PM1 (SCC*mec* VII,

accession number: AB462393, gene *ccrC8*). The *attR* site was identified 51 Kbp away from the *orfX* gene, Figure 2A, which deviates from typical SCC*mec* elements and therefore the *orfX* region was examined to find any additional *attR*-like site.

Within the 3' region of *orfX* an *att* site with sequence homology to the *attR SCCmec* typeVII of PM1, was identified. As there are two *ccr* regions within the 82B^{T} SCC*mec* it was proposed that the *att*R within the 3' of *orfX* (Figure 2, *att*R2) may be linked to the CcrC recombinase. Therefore, a search sequence: [at][at][at][ct][ct][ga][cg][atc][ta][ca][at][ta][ct][ac]act[ga][ga][tc]a, based on the *att*L sequence of SCC*mec* elements containing only the *ccrC* gene, was used to identify the corresponding *att*L site linked to *att*R2, *att*L2. This revealed only one potential *att*L2 site, located 16.8 Kbp upstream of the *mec* region (Figure 2, *att*L2). Sequence alignment of the *att*R/L sites of 82B^{T} with *att*R/L sites of *ccrAB* containing SCC*mec,* compared to the alignment of the *att*R/L2 sites of 82B^{T} versus those *att*R/L sites of *ccrC* containing SCC*mec,* suggest a varied *att* site for CcrAB/CcrC (Figure 3). Though it appears that the highly conserved central 8 bp sequence is consistent, there is notable variation between the *ccrAB* and *ccrC* associated *att* sites within those bases that flank the 8 bp region (Figure 3). Although the location of *att*L2 was identified with the predicted region, the only other potential *att*L site identified was *att*L', recently described for a highly conserved Type XI SCC*mec* found in *S. xylosus* 47-83 [30]. As with the *att*L' site identified for *S. xylosus* 47-83, the *att*L' of 82B^{T} lies downstream of the *lip* gene, however when compared to the *att*L of *ccrC* associated SCC*mec att*L sequences, it shares little similarity and lacks the central cysteine required for *att*B/*att*SCC recombination (Figure 3B) [27]. Indeed, the *att*L' of 82B^{T} shares more sequence similarity to that of *att*L2 from *S. scuiri* GVGS2 and the other *att*L sites related to CcrAB (Figure 3A). This suggests that *att*L2 is most likely linked with *att*R2, though its unusual genomic location within the SCC*mec* suggests various DNA recombination events may have occurred.

SCC*mec* include joining (J) regions, defined by the areas between the *orfX*, *ccr* and *mec* genes, with the SCC*mec* of 82B^{T} having 3 such J regions. The J1 region contains primarily hypothetical genes and the *ccrC* gene and shares 86% identity with the J1 region of *S. aureus* PM1 SCC*mec*. The J2 region upstream of *attL2*, contains genes similar to those of SCC*mec* type V, with the presence of type 1 restriction modification genes, although there is little similarity between the restriction modification genes of 82B^{T} and those present on SCC*mec* type V.

The J2 region downstream of *attL'* shows the greatest similarity to the SCC*mec* XI of LGA251, sharing 89% identity, with ABC transporters, genes associated with arsenic resistance and a lipase gene. Unlike the lipase gene in LGA251 the version in 82B^{T} appears to be intact. The J3 region is divergent from the rest of the SCC*mec* of LGA251, with the exception of a putative membrane protein and a putative DNA helicase protein. A notable feature of the J3 region is the presence of a lantibiotic biosynthetic cluster, that shares 90% nucleotide identity with one present on the plasmid pETB797 of *S. aureus* NRL 08/797 (accession number: KY436025). The cluster encodes two peptide homologues of Lacticin 3147, produced by *Lactococcus lactis*, which has been shown to be active against Gram-positive bacteria [31]. Within the cluster is also a gene encoding a homologue of LtnT which is required for the transport of the Lacticin 3147 peptides as well as unrelated peptides [32]. The J3 region also contains two ISL3-like transposases, with the majority of the other genes found within SCC*mec* from different staphylococci.

Antimicrobial susceptibly of *S. caeli* 82B^{T}

Using Vitek 2 isolate 82B^{T} was found to be susceptible to ciprofloxacin, daptomycin, gentamicin, linezolid, mupirocin, rifampicin, teicoplanin, tigecycline and vancomycin. The isolate was resistant to clindamycin, erythromycin, fusidic acid, tetracycline and trimethoprim. Genomic analysis revealed that 82B^{T} has the resistance genes *ermB, ermC, fusD, tetL* and *dfrK,* which is consistent

with the resistance profile of the isolate. With regards to β-lactam antibiotics it was resistant to benzylpenicillin (MIC ≥ 0.5 mg/L) and oxacillin (MIC 1-2 mg/L) but negative in the cefoxitin resistance screen. However, by disc diffusion it was resistant to cefoxitin and displayed a MIC of 3 mg/L when assessed by Etest. The isolate failed to grow on the MRSA screening agar MRSA Brilliance despite a high inoculum and the use of different incubation temperatures.

This study extends the known distribution and diversity of *mecC* genes in terms of country of isolation, sample type, staphylococcal host species, genomic context and allotypes, all of which are novel to the best of our knowledge. These highlight the complex epidemiology of this resistance determinant, especially among coagulase-negative staphylococci. It is interesting to note that the species most closely-related to *S. caeli*; *S. xylosus*, *S. saprophyticus* and *S. edaphicus* have all been reported to carry *mecC* or *mecC* allotypes which may indicate a prominent role for this group in the origins, evolution and epidemiology of *mecC* and SCC*mecC*.

Nucleotide accession numbers

The whole genome nucleotide sequences for *S. caeli* 82B^T have been deposited previously [18] in the NCBI database under accession numbers NZ_FMPG00000000 (assembly) and ERR473447 (sequence reads). The assembled SCC*mec* region of 82B^T generated in this work has been deposited under accession MH155596.

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Transparency declarations

Competing interests: none to declare.

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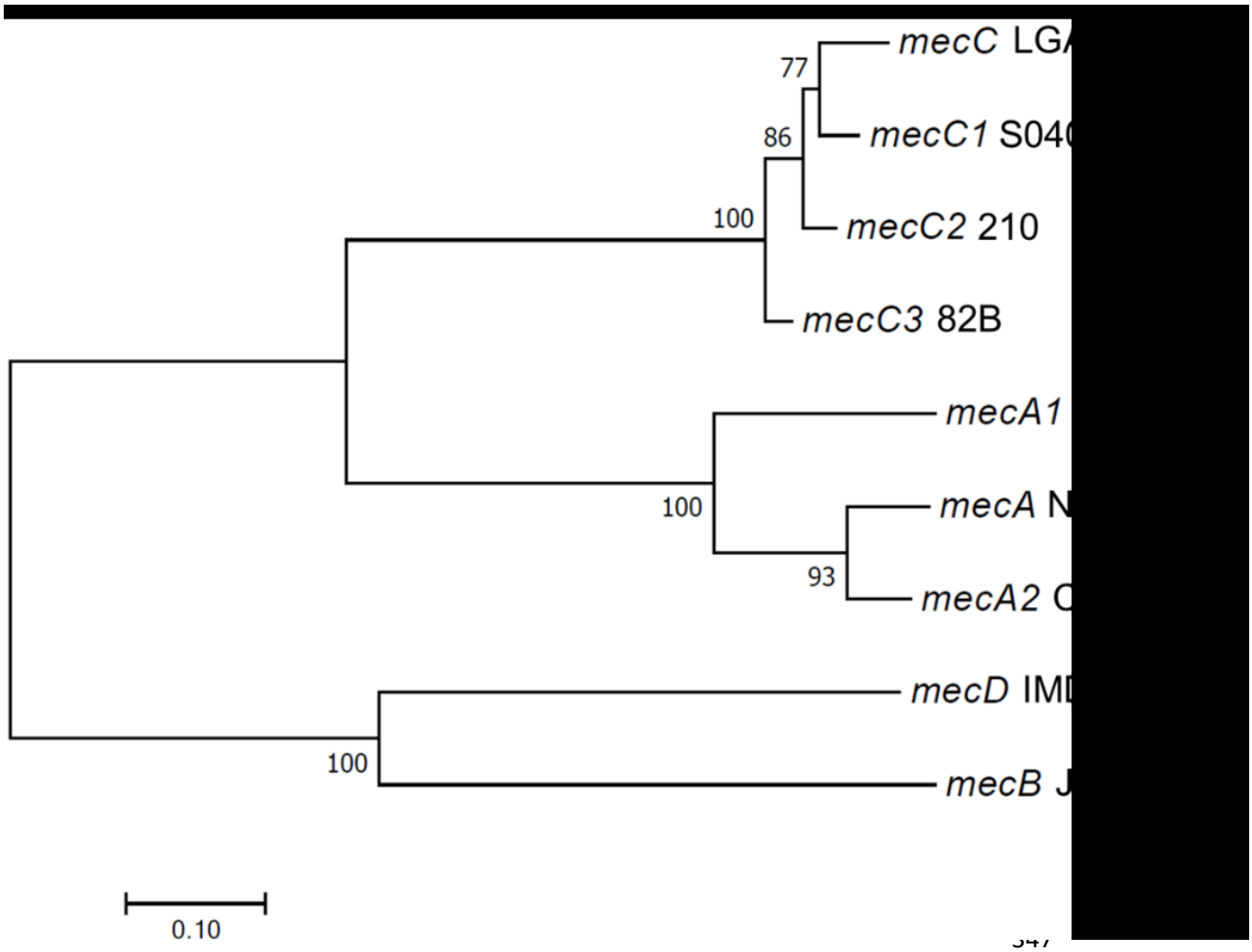
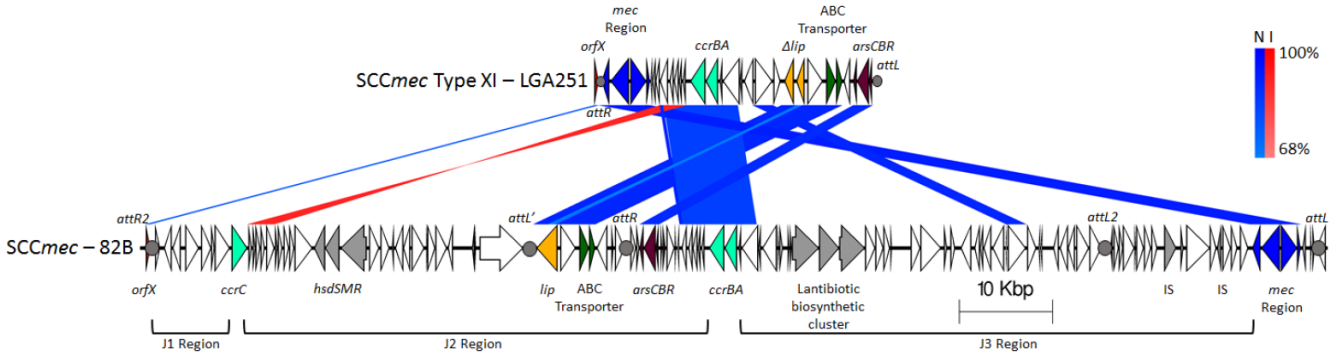


Figure 1. Phylogenetic relationship of representative *mec* allotypes. Nucleotide sequences were aligned using MUSCLE, with the Maximum Likelihood method, based on the General Time Reversible model, being used to build the tree. The highest log likelihood (-9785.4010) tree is shown. The percentage of trees in which the associated genes clustered together, based on bootstrapping with 500 replicates, is shown at the branches. A discrete gamma distribution was used to model site substitution rates, with branch lengths measured in the number of substitutions per site.



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359 **Figure 2.** Overview of *S. caeli* 82B^T SCC*mec*. SCC*mec* Type XI - LGA251 corresponds to *S.*
360 *aureus* LGA251, accession number FR821779, with SCC*mec* - 82B^T, corresponding to *S. caeli*
361 82B^T. Regions of homology are represented by bands connecting the two sequences, with the
362 percentage identity key shown on the right. Blue denotes normal sequence alignment (N); red,
363 denotes inverted sequence alignment (I). Key features associated with SCC*mec* elements, are
364 labelled. *att* sites are highlighted by filled circles and labelled above.

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Figure 3. Comparison of *att*R/L sites from *ccrAB* or *ccrC* containing SCC*mec*. Conserved nucleotide bases within the core 8 bp region, represented in black, bold font, are indicated by asterisk. The black triangle indicates the position of the central cytosine, thought to be essential for recombination between *attB* and *att*SCC [27]. The inverted repeats are marked by the underlined bases. (a) The sequences of known *attR* and *attL* sites associated with *ccrAB*, from *S. aureus* N315 (N315), NC_002745; *S. aureus* LGA251 (LGA251), FR821779; *S. xylosus* S04009 (S04009), HE993884; *S. sciuri* GVGS2 (GVGS2), HG515014; *S. aureus* ZTA09 (ZTA09), LK024544; were aligned and compared to those identified in *S. caeli* 82B^{T}. (b) The sequences of known *attR* and *attL* sites associated with *ccrC*, from *S. aureus* 85/2082 (85_2082), AB037671; *S. aureus* WIS (WIS), AB121219; *S. aureus* PM1 (PM1), AB462393; *S. aureus* TSGH17

(TSGH17), AB512767; *S. aureus* JCSC6082 (JCSC6082), AB373032; were aligned to *att*R2 and *att*L2 from *S. caeli* 82B^{T}.